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Emergence of Fluoroquinolone-Resistant *Haemophilus influenzae* Strains among Elderly Patients but Not among Children▽

Shin-ichi Yokota,[1] Yasuo Ohkoshi,[1,2] Kiyoshi Sato,[1] and Nobuhiro Fujii[1]*

Department of Microbiology, Sapporo Medical University School of Medicine, Chuo-ku, Sapporo 060-8556, Japan,[1] and Hokkaido Laboratory, SRL Co., Ltd., Chuo-ku, Sapporo 064-0919, Japan[2]

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We screened 457 *Haemophilus influenzae* strains isolated in Japan during 2002 to 2004 and identified 12 fluoroquinolone-resistant strains (2.6%). The resistant strains were divided into three genotypes (eight, three, and one of each type). These were isolated from patients over 58 years of age. Several fluoroquinolone-resistant clones appeared to have invaded the population of elderly patients in a particular area, Sapporo city.

Haemophilus influenzae, as well as *Streptococcus pneumoniae*, is a major causative agent of respiratory and otolaryngology infection, particularly community-acquired pneumonia in elderly persons and otitis media and sinusitis in children. Recently, there has been an increase worldwide in the prevalence of two types of β-lactam-resistant *H. influenzae* isolates: those that have acquired β-lactamases and those that carry mutations in the penicillin binding proteins. The latter are termed β-lactamase-negative ampicillin-resistant (BLNAR) strains. The recent increase in BLNAR strains has become a severe problem, leading to community-acquired infections (8, 19). Fluoroquinolone-resistant *H. influenzae* isolates have occasionally been reported worldwide (1, 4, 13, 15), but they have rarely been reported in Japan (9, 10). Fluoroquinolones are effective against the causative agents of atypical pneumonia, such as *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*, as well as those of typical bacterial pneumonia, *S. pneumoniae* and *H. influenzae*. The fluoroquinolones are considered to be candidates for the first choice of antimicrobial agents in cases of community-acquired pneumonia and otitis media in adults, so the emergence of fluoroquinolone-resistant *H. influenzae* and *S. pneumoniae* is a concern. Quinolone resistance is imparted by mutations in a particular domain referred to as the quinolone-resistance-determining region (QRDR) of one or both of the principal target enzymes, DNA gyrase (an A_2B_2 complex encoded by the *gyrA* and *gyrB* genes) and topoisomerase IV (a C_2E_2 complex encoded by the *parC* and *parE* genes).

In the present study, we screened and characterized fluoroquinolone-resistant *H. influenzae* strains isolated in clinical laboratories of general hospitals and a commercial clinical laboratory in Japan. Between 2002 and 2004, 457 clinical strains of *H. influenzae* were isolated at hospitals in the Hokkaido prefecture, Japan. The strains were collected and stored by Sapporo Clinical Laboratory, Inc. (Sapporo, Japan), Muroran General Hospital (Muroran, Japan), and Hokkaido University Hospital (Sapporo, Japan). Sapporo Clinical Laboratory, Inc., serves almost all areas of the Hokkaido prefecture. This study was approved by the institutional review boards of the organizations listed above. For each strain, we obtained information on the patient's age and sex, the clinical source, and the name of the city where the strain was isolated, in accordance with the Act on the Protection of Personal Information in Japanese law. The isolates were obtained from the following clinical specimens (with the numbers of samples in parentheses): sputum (190), rhinorrhea (178), nasal cavity (19), pharyngeal fluid (33), otopyorrhea (15), vaginal secreta (6), conjunctival discharge (5), bronchial lavage fluid (5), blood (2), urine (2), lochia (1), and cerebrospinal fluid (1). Cities where strains were isolated were Sapporo (228 strains), Muroran (112 strains), Asahikawa (61 strains), Tomakomai (23 strains), Obihiro (17 strains), Hakodate (14 strains), and Iwamizawa (2 strains). All isolates were grown at 37°C, in an atmosphere with 5% CO_2, on chocolate agar II (Nippon Beckton-Dickinson, Tokyo, Japan).

Determination of MICs of fluoroquinolones was carried out by a microdilution method on Mueller-Hinton medium supplemented with 5% horse blood with hemolysis and 15 μg/ml NAD, according to the standard method approved by the Clinical and Laboratory Standards Institute (CLSI) (Wayne, PA) (2). Levofloxacin (LVX) (Daiichi Pharmaceuticals, Tokyo, Japan), ciprofloxacin (CIP) (Bayer, Osaka, Japan), sparfloxaxin (SPX) (Dainippon-Sumitomo Pharma, Osaka, Japan), tosufloxacin (TSX) (Abbott, Osaka, Japan), gatifloxacin (GAT) (Kyorin Pharmaceuticals, Tokyo, Japan), and NM394 (active form of prulifloxacin [PUFX]) (Meiji Seika Kaisha, Tokyo, Japan) were kindly provided by the manufacturers. Determination of the MICs of other antibiotics was performed using a MICroFAST 4J panel (Dade Behring, Tokyo, Japan).

We carried out an initial screen for fluoroquinolone-resistant *H. influenzae* strains by using CIP and LVX. Twelve resistant strains were identified among 457 isolates (frequency of occurrence, 2.6%). All fluoroquinolone-resistant isolates were derived from patients that were over 58 years of age, and none were isolated from children (Table 1). The frequency of occurrence of fluoroquinolone-resistant strains among patients above 50 years of age was 10.2%. Similar age distributions were observed for patients with *S. pneumoniae* isolates in our previous reports (21, 22). We reported that fluoroquinolone-

* Corresponding author. Mailing address: Department of Microbiology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan. Phone: 81-11-611-2111. Fax: 81-11-612-5861. E-mail: fujii@sapmed.ac.jp.

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TABLE 1. Relationship between patient age and prevalence of fluoroquinolone-resistant *H. influenzae* clinical isolates

Patient age group (yr)	No. of strains	No. of resistant strains	Frequency of resistant strains (%)
0–9	285	0	0.0
10–19	10	0	0.0
20–29	7	0	0.0
30–39	20	0	0.0
40–49	11	0	0.0
50–59	26	1	3.8
60–69	41	2	4.9
70–79	42	5	11.9
≥80	15	4	26.7
Total	457	12	2.6

resistant *S. pneumoniae* strains were present in over 20% of elderly patients, but their overall frequency is low (2.4% of 670 strains tested) because they have not been identified in children. The most probable reason for this is that the use of fluoroquinolones other than norfloxacin has not been approved for children in Japan. In Japan, rare, sporadic occurrence (0 to 0.5%) of fluoroquinolone-resistant *H. influenzae* has been reported (6, 9, 23). In this study, fluoroquinolone-resistant strains were found with higher frequency (2.6%). The clinical samples obtained from adults were predominantly sputa, while those from children were mainly rhinorrhea. Ten of the resistant strains (83.3%) were isolated from sputa. All resistant strains were isolated in Sapporo city, which is the largest city in the Hokkaido prefecture, and 11 of 12 strains were isolated in the private clinical laboratory. However, these strains were isolated in more than one hospital in this city. More-detailed information on these hospitals and patients have not been available due to the Act on the Protection of Personal Information in Japanese law.

We determined the nucleotide sequences of the QRDRs of the quinolone target genes *parC*, *gyrA*, *parE*, and *gyrB* (Table 2). Genomic DNA was isolated from cells by using a DNeasy kit (QIAGEN, Hilden, Germany). PCR amplification was performed using HotStarTaq DNA polymerase (QIAGEN). The primer sets used to obtain DNA fragment of the *parC*, *gyrA*, *parE*, and *gyrB* QRDRs were previously described by Pérez-Vázquez et al. (14). Direct sequencing of the PCR products by the primers used for the PCR was carried out with an ABI PRISM3100 (Applied Biosystems, Foster City, CA). We examined the status of β-lactam resistance as follows. The presence of the genes for TEM-1 and ROB-1 β-lactamases and resistance-associated mutations in penicillin binding protein 3 (PBP3) was determined by PCR according to the method of Hasegawa et al. (5). In comparison to a fluoroquinolone-sensitive standard strain, Rd, all 12 resistant strains carried three to five mutations in these genes. We classified the 12 strains into three types, termed types I, II, and III, based on the patterns of mutations.

Type I (eight strains) showed extremely high MICs, which were four or eight times higher than cutoff values of nonsusceptible MICs proposed by CLSI (2). Type I commonly carried two mutations in ParC and GyrA and a single mutation in ParE. Mutations of Ser84Ile in ParC and Ser84Leu and

TABLE 2. Fluoroquinolone MICs and QRDR mutations in topoisomerase IV and DNA gyrase of fluoroquinolone-resistant *H. influenzae*

Type	Strain	Patient age (yr)	Sex[a]	Material	MIC (μg/ml)						Mutation(s)[b]			
					LVX	GAT	CIP	TSX	SPX	NM394	ParC	GyrA	ParE	GyrB
IA	SRI68	67	M	Sputum	32	8	16	≥32	16	4	Ser84Ile** (Asn138Ser)	Ser84Leu** Asp88Asn**	Asp420Asn*	
	SRI182	70	M	Sputum	16	8	8	≥32	8	2	Ser84Ile** (Asn138Ser)	Ser84Leu** Asp88Asn**	Asp420Asn*	
IB	SRI131	72	M	Rhinorrhea	16	8	16	≥32	8	2	Ser84Ile** (Asn138Ser)	Ser84Leu** Asp88Asn**	Asp420Asn*	
	SRI126	76	M	Sputum	16	8	16	≥32	8	2	Ser84Ile** (Asn138Ser)	Ser84Leu** Asp88Asn**	Asp420Asn*	
IC	SRI97	83	M	Sputum	16	4	8	≥32	8	2	Ser84Ile** (Asn138Ser)	Ser84Leu** Asp88Asn**	Asp420Asn*	
	SRI26	76	M	Sputum	16	8	16	≥32	8	2	Ser84Ile** (Asn138Ser)	Ser84Leu** Asp88Asn**	Asp420Asn*	
	SRI183	88	F	Sputum	16	8	16	≥32	8	2	Ser84Ile** (Asn138Ser)	Ser84Leu** Asp88Asn**	Asp420Asn*	
	SRI186	88	M	Sputum	16	8	8	≥32	8	2	Ser84Ile** (Asn138Ser)	Ser84Leu** Asp88Asn**	Asp420Asn*	
IIA	SRI38	84	M	Sputum	2	1	4	0.5	1	2	Gly82Cys* Ser84Arg**	Asp88Asn** Pro118Tyr?		Ser467Tyr*
	SRI181	58	F	Sputum	2	1	4	0.5	1	2	Gly82Cys* Ser84Arg**	Asp88Asn** Pro118Tyr?		Ser467Tyr*
IIB	SRI177	65	F	Conjunctival discharge	1	1	2	0.5	2	1	Ser84Arg**	Asp88Asn** Pro118Tyr?		Ser467Tyr*
III	HUI313	70	F	Sputum	0.5	0.5	1	0.25	1	0.5	(Asn138Ser)		Ser451Tyr? Pro452Leu?	

[a] M, male; F, female.
[b] **, resistance mutation previously seen in *H. influenzae*; *, resistance mutation previously seen in other bacterial species; ?, mutation with unknown contribution to resistance. Mutations in parentheses do not contribute resistance.

Asp88Asn in GyrA were previously reported to contribute to quinolone resistance in *H. influenzae* (4, 12, 14, 23). Asp420Asn in ParE corresponds to resistance-associated mutation sites in *Staphylococcus aureus* ParE (Asp432) and in *S. pneumoniae* ParE (Asp435) (16, 17). The Asn138Ser mutation in ParC was also found in susceptible *H. influenzae* strains (data not shown), which indicates that it does not contribute to fluoroquinolone resistance. Within the same genotype, strains had some different phenotypes, so we subdivided type I into types IA, IB, and IC (Tables 2 and 3). Strains SRI68 and SRI182 (type IA) carried the gene for TEM-1-type β-lactamase, did not have β-lactam resistance-associated mutations in PBP3, and had a high level of resistance to tetracycline and chloramphenicol (Table 3). Strains SRI131 and SRI126 (type IB) were BLNAR but showed ampicillin susceptibility, and they carried low-grade β-lactam resistance mutations in PBP3. The remaining four type I strains carried no β-lactam resistance mutations in PBP3 (thus, β-lactamase negative and ampicillin sensitive).

Type II (three strains) showed nonsusceptible MICs (≥2 μg/ml) to CIP and LVX MICs of 1 or 2 μg/ml, lower than the cutoff value of nonsusceptible MICs. Type II carried two mutations in GyrA, one or two mutations in ParC, and one mutation in GyrB (Table 2). Ser84Arg in ParC and Asp88Asn in GyrA are previously known mutations associated with quinolone resistance in *H. influenzae* (4, 12, 14, 23). Gly82Cys in ParC corresponds to Gly78 in *Escherichia coli* ParC and Gly85 in *Neisseria gonorrhoeae* ParC. Mutations of these residues have been shown to cause fluoroquinolone resistance (7, 11, 18). Ser467Tyr in GyrB corresponds to a resistance-associated mutation site in *Salmonella enterica* serovar Typhimurium ParE (Ser464) (3). It is unclear that Pro118Tyr in GyrA is involved in resistance. All type II strains carried the gene for TEM-1-type β-lactamase (Table 3). Type II strains were subdivided into type IIA (two strains) and IIB (one strain). Type IIA had low-grade resistance mutations in PBP3 and carried an additional mutation (Gly82Cys) in ParC. On the other hand, the two type IIA strains (SRI38 and SRI181) exhibited markedly different susceptibilities to cefaclor, cefotiam, and cefotaxime.

Type III (one strain) showed significantly higher MICs (1 μg/ml to CIP and 0.5 μg/ml to LVX) than other susceptible strains (<0.12 μg/ml), but these were lower than the nonsusceptible cutoff value. Type III carried a single mutation in ParC and two mutations in ParE. Asn138Ser is not related to resistance as described above. The type III strain contained only two mutations (Ser451Tyr and Pro452Leu) in QRDRs of ParE, and there is no evidence to date that these mutations are associated with fluoroquinolone resistance (Table 2). Additional studies are needed to determine whether mutations Pro118Tyr in GyrA (found in genotype II) and Ser451Tyr and Pro452Leu in ParC (found in genotype III) contribute to resistance.

These genotypes were confirmed by randomly amplified polymorphic DNA PCR (RAPD-PCR). RAPD-PCR was carried out as described previously (20). The sequences of the RAPD-PCR primers for *H. influenzae* were as follows: P4, 5′-AAGAGCCCGT-3′; P6, 5′-CCCGTCAGCA-3′; A04, 5′-ATCAGCGCACCA-3′; A05, 5′-AGCAGCGCCTCA-3′; A07, 5′-TGCCTCGCACCA-3′; and A08, 5′-GCCCCGTTAGCA-3′.

TABLE 3. Presence of the gene for TEM-1 type β-lactamase, PBP3 mutation phenotypes, and MICs for fluoroquinolone-resistant *H. influenzae*

Type	Strain	TEM-1 gene present[a]	Genotype[b]	MIC (μg/ml)												
				Ampicillin	Cefaclor	Cefixime	Cefditoren pivoxil	Cefotiam	Cefotaxime	Ceftriaxone	Cefozopran	Cefepime	Meropenem	Ampicillin-sulbactam	Amoxicillin-clavulanic acid	Clarithromycin	Tetracycline	Chloramphenicol	Rifampin
IA	SRI 68	+	BLPACS	≥8	4	≤0.25	≤0.03	2	≤0.12	≤0.12	≤0.5	≤0.25	≤0.12	1	≤1	16	≥8	≥8	≤0.5
	SRI182	+	BLPACS	≥8	2	≤0.25	≤0.03	1	≤0.12	≤0.12	≤0.5	≤0.25	≤0.12	1	≤1	16	≥8	≥8	≤0.5
IB	SRI131	−	Low BLNAR	0.25	2	≤0.25	≤0.03	1	≤0.12	≤0.12	≤0.5	≤0.25	≤0.12	≤0.5	≤1	32	≤0.5	1	≤0.5
	SRI126	−	Low BLNAR	0.25	2	≤0.25	≤0.03	1	≤0.12	≤0.12	≤0.5	≤0.25	≤0.12	≤0.5	≤1	32	≤0.5	≤0.5	≤0.5
IC	SRI97	−	BLNAS	0.25	1	≤0.25	≤0.03	2	≤0.12	≤0.12	≤0.5	≤0.25	≤0.12	≤0.5	≤1	32	≤0.5	≤0.5	≤0.5
	SRI26	−	BLNAS	0.25	1	≤0.25	≤0.03	1	≤0.12	≤0.12	≤0.5	≤0.25	≤0.12	≤0.5	≤1	32	≤0.5	≤0.5	1
	SRI183	−	BLNAS	0.25	1	≤0.25	≤0.03	1	≤0.12	≤0.12	≤0.5	≤0.25	≤0.12	≤0.5	≤1	32	≤0.5	≤0.5	≤0.5
	SRI186	−	BLNAS	0.25	1	≤0.25	≤0.03	1	≤0.12	≤0.12	≤0.5	≤0.25	≤0.12	≤0.5	≤1	16	≤0.5	≤0.5	≤0.5
IIA	SRI38	+	Low BLPACR	≥8	32	1	0.12	≥16	≥4	≤0.12	1	0.5	≤0.12	2	≤1	16	≤0.5	≤0.5	≤0.5
	SRI181	+	Low BLPACR	≥8	8	0.5	0.12	<0.12	≤0.12	≤0.12	≤0.5	0.5	≤0.12	2	≤1	16	≤0.5	≤0.5	≤0.5
IIB	SRI177	+	BLPACS	≥8	2	≤0.25	<0.03	1	≤0.12	≤0.12	≤0.5	≤0.25	≤0.12	1	≤1	16	≤0.5	≤0.5	≤0.5
III	HUI313	−	High BLNAR	0.5	2	≤0.25	0.06	2	≤0.12	≤0.12	≤0.5	≤0.25	≤0.12	≤0.5	≤1	32	≤0.5	1	≤0.5

[a] +, TEM-1 β-lactamase gene is present; −, TEM-1 β-lactamase gene is absent.

[b] Status of β-lactam resistance was determined by the PCR method as described in the text. BLNAS, β-lactamase negative, ampicillin sensitive; high BLNAR, β-lactamase negative, highly ampicillin resistant; low BLNAR, β-lactamase negative, weakly ampicillin resistant; BLPACS, β-lactamase positive, amoxicillin-clavulanic acid sensitive; low BLPACR, β-lactamase positive, weakly amoxicillin-clavulanic acid resistant.

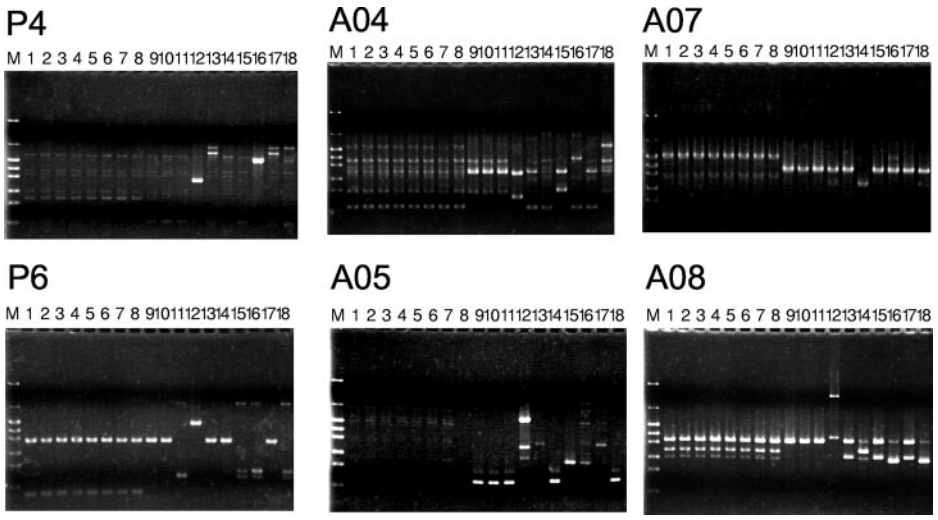

FIG. 1. RAPD-PCR analysis of fluoroquinolone resistance of *H. influenzae* clinical isolates. Lane M, size marker (pHY); lanes 1 to 8, genotype I strains (1, SRI68; 2, SRI182; 3, SRI131; 4, SRI126; 5, SRI97; 6, SRI26; 7, SRI183; 8, SRI186); lanes 9 to 11, genotype II strains (9, SRI38; 10, SRI181; 11, SRI177); lane 12, genotype III strain HUI313; lanes 13 to 18, strains susceptible to fluoroquinolones (13, SRI2; 14, SRI3; 15, SRI4; 16, SRI12; 17, SRI13; 18, SRI18). PCR primers used are indicated the top of each panel.

PCR amplification was performed using HotStarTaq DNA polymerase (QIAGEN) and GeneAmp PCR system 9700 (Applied Biosystems). DNA (20 ng) was used for each PCR. The PCR consisted of 95°C for 15 min, 35 cycles consisting of 94°C for 2 min, 38°C for 2 min, and 72°C for 2 min, and 72°C for 10 min. The products were analyzed by 1.5% (wt/vol) agarose gel electrophoresis. The results of RAPD-PCR analysis also correlated with this classification scheme (Fig. 1). Within each type, the RAPD pattern was indistinguishable, but there were clearly different RAPD patterns across types, except that one strain of type II gave a RAPD pattern that was different from those of the other two strains by primer P6 (Fig. 1, panel P6, lanes 9 to 11). This line of evidence suggested that the strains of each genotype were derived from a common ancestor.

We examined the resistance to other fluoroquinolones, such as TSX, SPX, GAT, and NM394 (active form of PUFX) (Table 1). The MICs of TSX, SPX, and GAT were similar to those of LVX and CIP. The order of resistance, from highest to lowest, was type I > type II > type III. The MICs of NM394 were similar for types I and II. With the exception of that of NM394, the MICs of all the fluoroquinolones tested against type I strains were extremely high. The MIC of NM394 was moderately high (1 to 2 μg/ml) for type I, similar to that for type II. For types II and III, the MICs indicated moderate resistance to all the fluoroquinolones, including NM394. One possible explanation for this is that the dominant target molecules of PUFX differ from those of the other fluoroquinolones. Another possibility is that there are other mechanisms of resistance that do not involve mutations in the QRDRs and that PUFX is refractory to these mechanisms of resistance. However, additional studies are needed to clarify this issue.

Lines of evidence indicated that the fluoroquinolone-resistant *H. influenzae* strains expanded clonally in a particular area, namely, Sapporo city. Strains that were assigned to the same genotype based on their mutation profiles and RAPD-PCR analysis had varied phenotypes. This suggested that a fluoroquinolone-resistant clone invaded in a city for a time required for the acquisition of genetic changes, such as the presence of the TEM-1 β-lactamase gene, mutations in PBP3, and resistance to tetracycline and chloramphenicol. Recently, Pérez-Vázquez et al. showed that fluoroquinolone resistance in *H. influenzae* occurs mainly in hypermutable strains (15). The fluoroquinolone-resistant strains found in the present study could be hypermutable, leading to phenotypic changes within genotypes. This clonal expansion of fluoroquinolone-resistant *H. influenzae* does not appear to be a transient outbreak, based on the fact that genetic variations were observed in the strains of the same genotype and the strains were not isolated from a single hospital.

The results of our current analysis are quite different from results of studies of *S. pneumoniae* (21, 22). Fluoroquinolone-resistant *S. pneumoniae* strains had marked differences in the patterns of mutation in their QRDRs and RAPD-PCR results and were isolated from various areas in the Hokkaido prefecture. Thus, it appears that fluoroquinolone-resistant *H. influenzae* strains expanded clonally, while fluoroquinolone-resistant *S. pneumoniae* strains were generated sporadically. Both strains of fluoroquinolone-resistant bacteria were found only in elderly patients because fluoroquinolones other than norfloxacin are not applicable to children in Japan.

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